IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of KEREM, B.
Serial No. 09/871,809
Filed: June 4, 2001
For: CONTROL OF GENE EXPRESSION

Group Art Unit: 1653
Examiner: Kam, Chih-Min

DECLARATION
under Rule 132

Commissioner of Patents and Trademarks
Washington, D.C. 20231

I, Hermona Soreq, an Israeli citizen residing at 14 HaMa'ayan St., Ein Kerem, Jerusalem, Israel, hereby declare:

1. I am currently Professor of Molecular Biology in the Department of Biological Chemistry and Vice Dean for R&D in the Faculty of Science of the Hebrew University of Jerusalem, Israel.

2. My *Curriculum Vitae* and list of publications is attached herewith as Annex "A". My fields of expertise include: alternative splicing in disease and aging.

3. I have read the patent specification entitled "Control of gene expression" which is U.S. patent application no. 09/871,809 (hereinafter "the application"). I have also read the claims of the application, and particularly claim 1.

4. The application [illegible] method of treating individuals suffering from a disease resulting from abnormal expression of genes caused by aberrant splicing in cells. This method comprises administering to cells of such individuals an effective amount of an alternative splicing factor (ASF), whereby said abnormal expression shifts towards normal expression of the gene.

5. Based on the description in the application, and based on the prior art, I believe that a person skilled in the art would be able to carry out the invention as defined in the claims.

6. My Declaration pertains to three major issues: a) identification of diseases that are due to aberrant splicing, b) identification of further ASFs and c) identification of treating conditions for treating such diseases using such ASFs.

7. As to diseases caused by aberrant splicing, this is a known definition of disease that enables any person skilled in the art to perform a simple search using publicly available data bases, and identify the diseases known to be caused by aberrant splicing. A sample of such a search carried out using the NCBI database, and limited to articles published prior to October, 1999, resulted in a list of publications, attached herewith as Annex "**B**", all describing diseases that are caused by aberrant splicing. Therefore, a person skilled in the art would have no problem in identifying such diseases. Additional diseases caused by aberrant splicing are described in my review paper, Meshorer E. and Soreq H., 2002, *Pre-mRNA splicing modulations in senescence*, Aging Cell, 1:10-16, attached herewith as Annex "**C**".

8. Alternative splicing factors (ASFs) are also a well-defined concept, well known to a person skilled in the art. Furthermore, the application discloses 7 different ASFs, all successfully used in order to correct splicing of mutated CFTR.

9. With respect to treating conditions, once the principle of the treatment has been revealed in the application, a person skilled in the art on reading the application, would understand how to apply the method defined in claim 1 to other diseases resulting from an abnormal expression of genes caused by aberrant splicing in cells, using routine experimentation as is well known in the field, such as expression vectors and proteins as discussed in the specification on page 6.

10. Therefore, it is my professional opinion that a person skilled in the art would know, based on the application, how to identify diseases caused by aberrant splicing and appropriate ASFs as well as how to use them in treatment of the diseases.

11. The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Jan. 29, 2003

Prof. Hermona Soreq

February 4, 2003

HERMONA SOREQ, PHD
PROFESSOR OF MOLECULAR BIOLOGY
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FAX: 972-2-642 9333; CELL PHONE: 972-54-337 866

Ph.D. The Weizmann Institute of Science (1976)
Thesis on: "Structure and Functions of Regions in mRNA as Probed by Purified Polynucleotide Phosphorylase".

MAJOR RESEARCH INTERESTS

Stress Responses, Antisense Technology, Acetylcholinesterase Biology, Molecular Neurobiology, Messenger RNA Studies
See Departmental website at http://sites.huji.ac.il/biolchem/soreq.html

PROFESSIONAL POSITIONS

2002 -	Vice Dean for R & D, Faculty of Natural Sciences, The Hebrew University
2000 -	Head, The Eric Roland Center for Neurodegenerative Diseases, The Hebrew University
1995 - 1999	Head, the Alexander Silberman Institute of Life Sciences, The Hebrew University
1992 -1995	Head, Dept. of Biological Chemistry, The Hebrew University
1989 - On	Professor of Molecular Biology, Department of Biological Chemistry, The Hebrew University
1986 - 1988	Associate Professor of Molecular Biology, Department of Biological Chemistry, The Hebrew University of Jerusalem.
1983 - 1986	Associate Professor, Neurobiology Department, Weizmann Institute
1979 - 1983	Senior Scientist, Neurobiology Department, Weizmann Institute
1977 - 1979	Fogarty Fellow, Department of Molecular Cell Biology, The Rockefeller University.
1976 - 1977	Scientist, Biochemistry Department, Weizmann Institute
1969 - 1971	Research Assistant, Biochemistry Department, Weizmann Institute, Rehovot.
1967 - 1969	Research and Teaching Assistant, Department of Biochemistry, Tel Aviv University.

AWARDS AND FELLOWSHIPS

2001	Honorary Professorship, The Maimonides University, Buenos Aires
2000	Research Prize by the Israeli Minister of Health
1999	Kay Prize for Innovative research, The Hebrew University
1996	Doctor of Philosophy honoris causa in Chemistry, University of Stockholm, Sweden.
1995	Visiting Professor, College de France, Paris.
1992	U.S. Army Science Award of excellence, Miami.
1990-	The Charlotte Slesinger Chair on Cancer Studies, The Hebrew University.
[illegible]	[illegible]
1985	Chancellor's Distinguished Lectureship, The University of California, Berkeley.
1982	Honorary Guest Lectureship, The European Society for Neurochemistry, Katania.
1980 - 1983	Charles Revson Career Development Chair, The Weizmann Institute.
1977	(1) B. de Rothschild Research Award. (2) H. Weizmann Postdoctoral Fellowship (declined). (3) Fogarty International Research Fellowship.
1974	Feinberg Graduate School Award, The Weizmann Institute.
1968	Shenkar Award, Tel Aviv University.

INTERNATIONAL PLENARY LECTURES (Past 5 years)

2003	Research Advisory Committee on Gulf War Veterans Illnesses of the Dept. of Veteran's Affairs Washington DC, USA (February)
2002	International Society of Developmental Neuroscience, Sidney, Australia (February) Alzheimer's Disease, Geneva, Switzerland (April) US Ministry of Defence (DARPA), Lexington, KY (April) American Society of Neurochemistry, Miami Beach, Florida (June) Genomics GIF meeting, Heidelberg (July). VII International Cholinesterase Meeting, Pucon, Chile (November)
2001	Mayo Clinic (January) Schering, Berlin (March) The Fifth International Conference on the Progress of Alzheimer's and Parkinson's

Disease, Kyoto, Japan (April)
The Maimonides University, Buenos Aires, Argentina (May)
The Gordon Research Conference on Stress induced gene Expansion, Conneticut (July)
University of Buenos Aires, Argentina (August)
Ageing and Dementia, Graz (September)
The Society of Controlled Release, Paris, France (July)
The Karolinska Institute, Stockholm, Sweden (September)
The British Society for Cell-Matrix Associations, Newcastle (September)
The German Society for Biochemistry and Molecular Biology, Munich, Germany (October)
Medizinsche Gesellschaft Lecture, Erlangen, Germany (October)
The University of Heidelberg, Germany (December)
The Winter Series on Aging, Galveston, Texas (March).
CARES visiting lectureship, Chicago, Illinois (April).
Japanese Society of Neurology, Tokyo (May).
The US Society of Neuroscience, Miami, Florida (October).
The Herman Niemeyer Plenary Lecture, Chilean Society of Biology, Pucon, Chile (November).

1998 The Sixth International Congress on Psychiatric Genetics, Bonn, Germany.
The Fourth Congress of the Asian Physiological Societies, Brisbane, Australia.

INTERNATIONAL SOCIETIES AND COMMITTEES

2000-2005	Council Member, The International Society for Developmental Neuroscience
1999-2001	President, The Israel Society for Biochemistry and Molecular Biology
1999-2002	Member, Federation of European Neuroscience (FENS) Course Committee
1996-2000	Member, EMBO Long term fellowships committee
1996	Observer, European Community Committee on Biotechnology
1993-On	Steering Committee, European Neurobiology Network (ENN).

Council Member: European Society of Neurochemistry (ESN,1992-1996), International Brain-Research Organization (IBRO, 1994-1996), International Society of Neurochemistry (ISN, 2001-2004), International Society of Developmental Neuroscience (ISDN, 2002-2005).

Member: European Molecular Biology Organization (EMBO), Human Genome Organization (HUGO), Federations of American and Israeli Societies for Experimental Biology (FASEB, FISEB), American Societies for Neuroscience & for Pharmaceutical & Experimental therapeutics, Society of Controlled Release.

NATIONAL COMMITTEES

2001-	Member, Israel Interdisciplinary Center for Neuronal Computation (ICNC)
2001-	Bioethics Committee, Israel Academy of Sciences and Humanities
2000-2002	Head, Infrastructure Advisory Committee to the Minister of Science
1998-2002	FIRST Committee for interdisciplinary research, the Israel Academy of Sciences and Humanities.
1997	Scientific Visiting Committee, The Ben Gurion University Department of Biology
1995-	Consulting Committees to the Chief Scientist at the Ministry of Health
1995-1999	Biotechnology Committee, assigned by the Minister of Science
1994-1999	Human Genome Committee, assigned by the Israel Academy of Sciences and Humanities

BOARDS OF DIRECTORS

2002-	FIRST fund, the Israel Academy of Sciences and Humanities
1997-2002	The Landau Fund, Mifal HaPayis
1998-	Machteshim Agan (MA Industries)

HEBREW UNIVERSITY COMMITTEES

2002-	Senate Representative in the Board of Governors.
2002-	Board of Directors
2002-	Co-Chair, International Work Group on Stress Responses, The Institute for Advanced Studies (Together with D. Engleberg).
2001-	Genomics Committee
1995-1999	Head, The Life Sciences Institute Committees (R&D, Organization)
1995-2000	Senate Representative in the Board of Governors
1994-1995	Head, Committee for Life Sciences recruitments
1990-1991	Ph.D. Studies Committee
	Infrastructure Committee

ISRAELI UNIVERSITY COMMITTEES

2001-	Visiting Committee, Ben-Gurion University's Biology Dept. (with I. Chet)
2002-	Ramot Academic Committee for Biomedical Research, Tel Aviv University

INTERNATIONAL CONFERENCE COMMITTEES

Head, Program Committees:

2002	Co-Chair, Joint Meeting on Molecular Neuroscience, Heidelberg Univeristy-Hebrew University
2002	Joint FASEB - IUBMB Conference, Istanbul
1999	Co-Chair, Program Committee, IBRO Conference, Jerusalem.
1998	New Jersey-Israel Conference on Biotechnology, New Jersey.
1994	Tenth Biannual ESN Conference, Jerusalem.
1993	Bath Sheva de Rothschild workshop on Genome Diversity, Kfar Saba.
1991	Eighth Biannual meeting, European Society of Developmental Biology, Jerusalem.
1991	Fourth Biannual meeting, European Oocyte club, Eilat.

Member of Program Committees:

2003	6th International Conference on Alzheimer's and Parkinson's Disease
2002	7th International Symposium on Advances in Alzheimer Therapy, Geneva.
2002	11th International symposium on Cholinergic Mechanisms, St. Maurtiz.
2000	6th International Symposium on Advances in Alzheimer Therapy, Stockholm.
1998	Biannual ESN Conference, St. Petersburg.
1997	International ISN/ASN meeting, Boston.
1997	International Alzheimer and Parkinson's Disease Meeting, Eilat.
1996	Israeli Biotechnology toward the year 2000, Rehovot.
1991	International Society of Neurochemistry (ISN), Montpellier.

EDITORIAL WORK

1991 - 1997	Deputy Chief Editor for Minireviews, Journal of Neurochemistry
1991 - 1997	Associate Editor, Molecular and Cellular Neurobiology
1991	A special issue on cholinesterases, Molecular and Cellular Neurobiology
1984	Molecular Biology Approach to the Neurosciences. IBRO Handbook Series, methods in the neurosciences. Vol. 7. John Wiley and Sons (London, New York).

Membership in Editorial Boards

2001-	NeuroMolecular Medicine
2000-2003	Neurobiology of Disease
2000-	Israel Medical Association Journal
1999-	European Journal of Biochemistry, Journal of Applied Toxicology
1997-	International Journal of Molecular Medicine
1995 -1998	Science Spectra
1994 -	CNS Drug Reviews
1993 -	Antisense Research and Drug Development
1986	Journal of Molecular Neuroscience

TEACHING ASSIGNMENTS

Hebrew University:

2002	ETGAR (Honors) students, 1st year.
1999 – on	Selected Topics in Life Sciences Research, 1st year Biology undergraduates.
1994 - on	Molecular Processes in the brain, 3rd year and Research students in Biology.
	Biochemistry, a laboratory course for 3rd year Biology undergraduate students.
1991 - 1995	Recombinant DNA in Biological Research, a laboratory course for honours undergraduate students.
1987 - on	1. Introduction to Molecular Biology Course, 2nd year Biology and Biochemistry students. 2. Intensive Laboratory Workshop on Eukaryotic Gene Expression: From transgene to organism; post-graduate students

International:

2001, 2003	Teacher, ISN summer course, Buenos Aires, Hong-Kong
1998	Co-organizer, EMBO practical course on Molecular Neurophysiology, Jerusalem.
1984	Organizer, EMBO practical course on Molecular Neurobiology, Rehovot.

STUDENTS AND POST-DOCTORAL FELLOWS

A. **M.Sc. Students**

1. **Daniel Eliyahu**, 1980-1982.

Altered Ontogenesis of Specific proteins in agranullar cerebellar cortex.

2. **Ruti Parvari**, 1980-1983.
Biosynthesis of acetylcholinesterase in rat brain and embryonic *Torpedo* organ as studied by the expression of its scarce mRNA species in microinjected *Xenopus* oocytes. (Cosupervisor: I. Silman).
Feinberg Graduate School Award, 1982.

3. **Anat Safran**, 1981-1983.
Variations in translatable mRNAs during development of the rat cerebellum.
Feinberg Graduate School Award, 1983.

4. Margit **Burmeister**, 1982-1984.
Studies on the biosynthesis of epidermal growth factor in microinjected *Xenopus* oocytes. (Cosupervisor: J. Schlesinger) *Minerva Fellowship.*

5. **Adi Avni**, 1983-1984.
Isolation and partial characterization of a human acetylcholinesterase gene identified by homology to the *Drosophila* gene.
Feinberg Graduate School Special Award, 1984.

6. **Ronit Zamir**, 1986-1988.
Chromosomal mapping of human cholinesterase genes.

7. **Shlomo Seidman**, 1987-1989.
Expression and tissue-specific processing of cloned human butyrylcholinesterase in mRNA injected *Xenopus laevis* oocytes.

8. **Efrat Lev-Lehman**, 1990 - 1991.
Localization of cholinesterase mRNA transcripts in the mammalian brain.

9. **Nilli Galyam**, 1997-1999.
Neuronal and hematopoietic consequences of antisense acetylcholinesterase suppression. Pollack Award,
1998. *Wolf Award, 1999.*

10. **Nadav Livny**, 1998-1999.
Genetic and epigenetic contributions toward anticholinesterase insults.

11. **Nelly Gluzman**, 1997-2000.
Site-directed mutagenesis approach to drug interactions of human cholinesterases.

12. **Danijel AlBajari**, 1997 – 2001.
Structure-function relationships between acetylcholinesterase and presenilins.
Boehringer-Ingleheim fellowship, 1998-2000.

13. **Alastaire Grant**, (BSc University College, London), 1999 – 2001.
Functional polymophisms in the ACHE locus.*UK Friends of HUJ fellowship, 1999-2001.*

14. **Liat Ben Moyal**, (BSc Ben Gurion University), 2001-
Epigenetic risks associated with ACHE gene expression. *Hazelkorn fellowship, 2001-2002.*

15. **Boris Bryk**, (The Hebrew University), 2002.
Stress-associated role(s) of the splicing factor SC35.

16. **Deborah Toiber**, (The Hebrew University), 2002.
Novel human and murine Acetlycholinesterase variants.

17. **Adi Gefen** (co-superviser: Raz Yirmia, Dept. of Psychology, The Hebrew University)
[illegible] cytokine gene expression.

B. **Ph.D. Students (and thesis topics)**

1. **Averell Gnatt**, 1985-1990.
Structure-Function relationships in human cholinesterase genes and their protein products.
Landau Award, 1990

2. **Revital Ben-Aziz Aloya**, 1989 - 1993.
Post-transcriptional regulation of the human acetylcholinesterase gene. Landau Award, 1991.

3. **Gal Ehrlich**, 1989 - 1993.
Congenital and acquired modulations in the human cholinesterase genes in tumor and healthy tissues. *Golda Meir Award*, 1989, *Pollack Award, 1990.*

4. **Shlomo Seidman**, 1990-1994.
A morphogenic Role for acetylcholinesterasc: Heterologous Expression Studies in microinjected embryos of Xenopus laevis. *Magna Cum Laude. Landau Award, 1995.*

5. Yael **Loewenstein-Lichtenstein**, 1990 - 1996.
Molecular dissection of active domains in human cholinesterases.
Pollack Award, 1991, Landau Award, 1994, Human Frontiers Post-doctoral Fellowship, 1996- 1998.

6. Rachel **Beeri-Leibson**, 1991-1997.
Human acetylcholinesterase expression in transgenic mice: An approach to the molecular control of cholinergic responses. *European Neurobiology Network Award, 1995, Lady Davies Post-doctoral Fellowship, 1997.*

7. **Efrat Lev-Lehman**, 1992 - 1997.
Developmental role(s) of acetylcholinesterase revealed by multileveled modulations of ACHE gene expression.
Golda Meir Award, 1990. B. de Rothschild Post-doctoral Fellowship, 1997-1999.

8. **Mirta Grifman**, 1993 – 1998.
Modulation of cholinergic signalling by Antisense Technology.
ISN Travel Fellowship, 1995. Mexican HUJ Friends Fellowship, 1996.

9. **Meira Sternfeld**, 1992 –1999.
Structural and catalytic functions of alternative human cholinesterase variants in native and transgenic systems.
Pollack Award, 1993. Lady Davis Post-doctoral Fellowship, 1999.

10. **Daniela Kaufer**, 1994 –1999.
Molecular mechanisms underlying cholinergic stress responses in mammals.
Pollack Award, 1996. ISN Travel Fellowship, 1997. EMBO Post-doctoral Fellowship, 1999 (declined). Human Frontiers Post-doctoral Fellowship, 1999. LSRF Post-Doctoral Fellowship, 2002.

11. **Michael Shapira**, 1994 – 2000.
Neurogenetics approach to the transcriptional control of acetylcholinesterase production.
Pollack Award, 1995. *Maria-Ascoli Award, 1999. Deans' Post-doctoral Fellowship, 2001.*

12. **Osnat Cohen**, DVM (HUJ), 1997-
Dissection of Acetylcholinesterase Contributions toward the Cholinergic Control of Mammalian Behavior (co-supervisor: R. Yirmiya, Psychology). *ASPECT Fellowship and Best Paper Award, 2000.*

13. **Noa Farchi**, 1998-
The roles of AChE splice variants in neuronal and muscle function: transgenic engineering approach.
(co-supervisor: B. Hochner, Neurobiology). *Dean's Award, 1999, 2000, Pollack Award, 1999.*

14. **Inbal Mor**, M.Sc. (HUJ), 1998-
Morphogenic functions of acetylcholinesterase variants in terminal differentiation.
Dean's Award, 2000. Pollack Award, 2001.

15. **Ella Sklan**, M.Sc. (Ben Gurion University), 1999-
Genetic Engineering Approach to acetylcholinesterase interactions with partner proteins.

16. **Eran Meshorer**, M.Sc. (HUJ), 1999-
Delayed molecular consequences of nervous system stress responses: from DNA microarrays to altered neurotransmission pathways. *ISN Travel Award, 2001. Lichtenstein Award, 2001, ICNC PhD fellowship, 2002.*

17. **Marjorie Pick**, M.Sc. (University of Melbourne), 2000-
Hematopoietic functions of acetylcholinesterase-derived C-terminal peptides. (co-supervisors: A. Eldor and E. Naparstec, TAU).

18. **Irit Shapira**, B.Sc. (in psychology, HUJ), 2000-
Molecular mediators of cognitive responses to psychological and physiological stress. (co-supervisor: R. Yirmiya, Psychology).

15. **Tama Evron**, B.Sc. (HUJ) 1999-
Acetylcholinesterase-associated modulations of immune responses.

16. **Akiva Korn**, (BSc Bar Ilan University), 1999 –
[illegible] and anticholinesterase exposures
Co-Supervisor: Alon Friedman (Ben Gurion University).

17. **Dror Gal**, M.Sc (TAU), 2002-
Molecular neurogenetics of suppressed Acetlycholinesterase gene expression

18. **Erez Podoly**, M.Sc. (HUJ), 2002
Structural implications of AChE's protein-protein interactions
Co-Supervisor: Oded Livneh

C. **M.D. Ph.D. Students (and thesis topics)**

1. **Patrick Dreyfus**, M.D. (The University of Paris), 1986-1989.
Multileveled regulation of the human cholinesterase genes and their protein products.
INSERM exchange visitor.

2. **Yaron Lifson-Lapidot**, M.D. (Ben-Gurion University), 1989-1991.
Elements of cholinergic signalling in Hematopoiesis.
Levi Eshkol Fellowship

3. **Daniel Grisaru**, M.D. (Tel Aviv University), 1996 – 2001.
Morphogenic Function(s) of Acetylcholinesterase variants and fragments thereof in normal and pathological
development.
(co-supervisor: A. Eldor, TAU). *Meirbaum award, 1998.*

4. **Asher Salmon**, M.D. (Technion), 1998 – 2001.

Genetic Engineering approaches into cholinesterase interactions with Xenobiotic agents.
ENN short-term fellowship, 1998. Barclays Post-doctoral Fellowship, 2001.

5. **Chava Perry**, M.D. (HUJ), 2000-
Molecular mechanisms underlying acetylchoinesterase associated tumorogenesis.
(co-supervisor: the late A. Eldor, TAU)
Meirbaum Award, 2000.Long-term Ministry of Health Fellowship, 2001.
6. **Michael Levy**, M.D./Ph.D. program, (HUJ), 2001-
Antisense modulations of cognitive processes.
Co-Supervisor: Hagai Bergman (Faculty of Medicine).
7. **Rinat Kahat**, M.D. (Technion), 2001-
Acetylcholinesterase in retinal functioning.
(co-supervisor with Ido Perlman, Rapaport Institute).

D. M.D. Basic Research Fellows

1. **Nissim Razon**, M.D., (Tel Aviv University) 1982.
Selective Impairment of Gene Expression in Human Brain Tumors.
Bornstein Award, 1982.
2. **Avi Matzkel**, M.D., (Tel Aviv University), 1983.
Polymorphism of acetylcholinesterase in fetal human tissues.
3. **Gustavo Malinger**, M.D., (Tel Aviv University), 1986.
The Expression of Human Cholinesterase Genes in Normal and Malignant ovary.
Israel Fertility Association Award, 1986.
4. **Eduardo Schejter**, M.D., (Tel Aviv University), 1987.
Expression of human cholinesterase genes in muscle.
5. **Ari Ayalon**, M.D., (Tel Aviv University), 1988.
Expression of human cholinesterase genes in fetal and neoplastic brain tissues as detected by in situ hybridization.
6. **Adrian Katz**, M.D., (Tel Aviv University), 1989.
Spermatogenic expression of human cholinesterase genes.
7. **Asher Salmon**, M.D., (Technion, Haifa), 1997.
Variable interactions of alternative cholinesterases with heroin derivatives.
8. **Tatiana Wender**, M.D., (Ben Gurion University), 2001.
ACHE promoter polymorphisms in occupationally induced Parkinson's Disease.
9. **Amir Dori**, M.D., PhD (Ben Gurion University), 2002.
Neurogenic Functions of Embryonic AChE.

E. Post-Doctoral Fellows

1. **Sherena Cedar**, Ph.D. (in Immunology, London University) 1983 - 1984.
The expression of cholinesterase in inducible human erythroleukaemic cells lines.
EMBO Post-Doctoral Fellowship.
2. **Catherine Prody**, Ph.D. (in Biochemistry, University of California, Berkeley) 1984 - 1988.
Molecular cloning of human cDNA sequences coding for cholinesterases.
MDA Fellow.

Expression of cloned acetylcholinesterase cDNA in microinjected *Xenopus* oocytes.
Levi Eshkol Fellowship.

4. **Lewis Neville**, Ph.D. (in Neurobiology, University of Southhampton) 1989-1991.
Expression of natural and site-directed cholinesterase variants in microinjected Xenopus oocytes.
Golda Meir Fellowship.
5. **Averell Gnatt**, Ph.D. (in Biochemistry, Hebrew University), 1991-1992.
site-directed mutagenesis of recombinant human butyrylcholinesterase.
Honorary ESN Lecturer, 1992, Dublin
6. **Rachel Karpel**, Ph.D., (in Ecology, Hebrew University), 1991 - 1994.
Cholinergic signalling and cell division control.
ICRF Fellowship.
7. **Mikael Schwarz**, Ph.D. (in Botany, Hebrew University), 1992- 1994.
Molecular dissection of catalytic events of cholinesterases.
Levy Eshkol Fellowship.
8. **Ellen Chaikin**, Ph.D. (in Developmental Biochemistry, Hebrew University), 1993.
Expression of cholinesterases in osteogenesis.
Golda Meir Fellowship.
9. **Christian Andres**, M.D., Ph.D (in Neurochemistry, University of Strasbourg), 1993 - 1995.

Transgenic expression of human acetylcholinesterase in murine nervous system.
INSERM & NCRD-Israel Ministry of Science Exchange Fellowships.

10. **Alon Friedman**, M.D., Ph.D. (in Neurobiology, Ben Gurion University), 1996-1998.
Electrophysiological and molecular mechanisms underlying cholinergic hyperexcitation.
Smith Psychobiology Post-doctoral Fellowship. Foulkes Prize, 1997. Teva Prize, 1997.

12. **Ron Broide**, Ph.D. (in Neurobiology, University of CA., Irvine), 1995-1997.
Photoreceptor degeneration in ACHE transgenic mice.
Valazzi - Pikovsky Fellowship.

13. **Christina Erb**, Ph.D. (in Pharmacology, University of Mainz), 1999-2000.
Transgenic approach to the molecular neuropharmacology of cholinergic transmission.
Long-Term Minerva Fellowship.

14. **Klara Birikh**, Ph.D. (in Molecular Biology, Moscow University), 1999 – 2000.
Conditional antisense suppression of neuronal acetylcholinesterase production.
Long-Term EMBO Fellowship.

15. **Cesar Flores Flores**, Ph.D. (in Biochemistry, Univ. of Murcia, Spain) 1999 –
Combinatorial search for monoclonal anti-AChE antibodies.
Golda Meir Fellowship (declined); *Long-term FEBS Fellowship.*

16. **Amir Dori**, MD, PhD (in Physiology, BGU), 2002-
Neurodevelopmental implications of acetylcholinesterase gene expression.
Smith Psychology Post-doctoral Fellowship.

F. MAJOR EXTRAMURAL COLLABORATIONS

1. **Charles J. Arntzen**, Chariman, Department of Plant Biology, and Tsafrir Mor, Research Associate, Arizona State University, Member, US President's Advisory Council on Science.
Human acetylcholinesterase production in transgenic tomatos (DARPA grant 2001-2004).

2. **Fritz Eckstein**, The Max Planck Institut for Experimental Medicine, Gottingen, W. Germany.
Use of Antisense oligodeoxynucleotides for the *in vivo* modulation of cholinergic signalling and survival.
(Ministry of Science grant, 1991 - 1994; GIF grant, 1994-1997;
Joels Visiting Professor November 1997 - February 1998, Hebrew University of Jerusalem).

3. The late **Amiram Eldor**, Chairman, Department of Hematology, Sourasky Medical Center, Tel Aviv.
Hematopoietic function(s) of human acetylcholinesterase variants.

4. **Alon Friedman**, Department of Neurosurgery, Soroka Medical Center, Beersheva.
US Army Medical Research and Development Grant, 1999-2004

5. **Israel Hanin**, Chairman, Department of Pharmacology & Experimental Therapeutics, Loyola University, Chicago. Cholinotoxic effects on brain-region specific alterations in G,C-rich transcripts. *Lady Davies Fellow, 1993. Smith Psychobiology Fund, 1993.*

6. **James Patrick**, Vice President and Dean of Research, Baylor Medical School, Houston, Texas.
Transgenic modulations of cholinergic functions. (BSF Grants, 1989-1992, 1993-1996, 1997-1999, 2000- 2002).

7. **Gene Robinson**, Dept. of Entomology, University of Illinois, Urbana, IL U.S.A.
Molecular Genetics approach to Honey Bee Acetylcholinesterase. *Fullbright Fellow*, 1996. *Smith Psychobiology Fund*, 1996.

8. **Raz Yirmiya**, Dept. of Psychology, HUJ.
Cholinergic modulations of mammalian behavior

9. Haim **Zakut**, The Sackler Faculty of Medicine, Tel Aviv University.
Expression of human cholinesterase genes in fetal development and in neoplastic tissues (Academy grant, 1994-1996: Ministry of health grant, 1995-1996).

PATENT APPLICATIONS

Title	Application/ Pat. No	Application Date	Current Status	Yissum (Luzz.)
Genetically Engineered Human Cholinesterases	IL Priority	21/03/89	Renewed 03/02/02	1961.00 (89703)
	EU 0 388 906	20/03/90	Granted 14/06/95	1961.03 (90105274)
	F 0 388 906	14/06/95	Granted 14/06/95	1961.04
	CH 0 388 906	14/06/95	Granted 14/06/95	1961.05
	GB 0 388 906	14/06/95	Granted 14/06/95	1961.06 (90105274.6)
	DE 690 20018.8	14/06/95	Granted 14/06/95	1961.07
	US 5,595,903.	02/08/93	Granted 21/01/97	1961.08 (6321) (08/111,314)
Transgenic Animal Assay System for Anticholinesterase Substances	US 5,932,780	09/01/95	Granted 03/08/1999	2098.01 (08/370, 156 [814095])
	PCT/US 95/02806	28/02/95	Published 31/8/95	2098.02 (6312)
	EU 95913580.7	28/02/95	Filed	2098.03
	US 6,025,183	06/03/97	Granted 15/2/00	2098.04
	US 09/310,638	12/5/99	Examined	2098.05
Therapies Utilizing Antisense Oligonucleotides and Butyryl-Cholinesterase Inhibitors	US	12/12/96	Provisional	(60/035,266)
A method and composition for enabling passage through the blood-brain barrier	PCT/US97/21696	20/11/97	Published 28/5/9	2151.02 (6439)
	US 6,258,780	20/11/97	Granted 10/7/01	2151.03
	IL 129990	20/11/97	Filed	2151.04
	AUS 53642/98	20/11/97	Granted 10/7/01	2151.05
	CAN 2272280	20/11/97	Filed	2151.06
	JAP 10-523989	20/11/97	Filed	2151.08
Method of Screening For Genetic Predisposition to Anticholinesterase Therapy	PCT/US9 00322	11/1/96	Published 18/7/96	2207.01 (6785)
	US 08/370,204	09/01/95	Allowed partially appeal 13/10/99	2207.03
	CAN 2,209,683	11/1/96	Filed	2207.04
	JAP 8-521788	11/1/96	Published 24/11/98	2207.06
	US 5,807,671 Priority	9/1/95	Granted 15/9/98	2207.00
	US 6,326,139	09/01/95	Granted 4/12/2001	2207-05

Synthetic antisense oligodeoxynucleotides targeted to human ACHE (Title as US patent)	PCT/US 97/23598	12/12/97	Published 18/6/98 WO98/26062	2304.01 (6707)
	US 6,121,046	12/12/97	Granted 19/09/00	2304.02
	IL 130162	12/12/97	Filed	2304.03
	AU 727611	12/12/97	Granted 14/10/00	2304.04
	CAN 2,274,985	12/12/97	Filed	2304.05
	EP 0951536	12/12/97	Published 15/9/99	2304.06
	JP 10-527069	12/12/97	Filed	2304.07
	US 09/572,630 Cip		Examined	2304.08
Synthetic antisense oligodeoxynucleotides and pharmaceutical composition containing them	PCT/EP 93/00911	15/4/93	Published 28/10/93 WO93/21202	2042.01 (6692)
	JAP 517984/93	15/4/93	Abandoned	2042.02
	EP 0636137	15/4/93	Granted Validated in FR,DE &UK	2042.03
	US 5,891,725	1/12/94	Granted 6/4/99	2042.05
	CAN 2,118,235	15/4/93	Filed	2042.06
Cip title: *Synthetic antisense oligodeoxynucleotides targeted to ACHE*	US 6,110,740 Cip patent	2/5/98	Granted 29/8/00	2042.07
Title: *Deoxyoligonucleotides and pharmaceutical compositions containing the same*	IL 101600 (Priority)	15/4/92	Granted 30/5/00	2042.00
Methods and compositions for the treatment of injury to the central nervous system	PCT/US 98/04503	6/3/98	Published 11/9/98 WO98/39486	2325.01 (6720)
	AUS 64521/98	6/3/98	Abandoned	2325.02
	CAN 2,283,068	6/3/98	Filed	2325.03
	US 09/380532	6/3/98	Examined	2325.04
	EP 98910229.8		Abandoned	2325.05
Diagnostic uses of antibodies against AChE or C-terminal peptides thereof	IL 130225 Priority	31/05/99	Examined	2463.00 (8248)
	PCT/IL 00/00312	31/5/00	Published 2/12/00 WO00/73343	2463.01
	US	31/5/00		2463.02
	EU	31/5/00		2463.03
	CA	31/5/00		2463.04
	IL	31/5/00		2463.05
Pharmaceutical compositions comprising AChE AS-ODNs for the treatment of muscular and neuromuscular disorders	IL 132972	16/11/99	Filed	2428.00 (6711)
	PCT/IL 00/00763	16/11/00	Filed	2428.01
Human antibodies to specific AChE variants and their use as diagnostic agents for cholinergic neurodeterioration processes	IL 140071	4/12/00	Filed	2557.00 (12261)
	PCT/IL 01/00464	19/06/01	Filed	2557.01
Antisense oligonucleotide against human AChE and uses thereof	IL 143379	24/05/01	Filed	2584.00 (13122)
System and method for assaying drugs	US 60/247,970 PCT/IL01/01051	14/11/00 14/11/01	Exhausted filed	2547 (13825)
Acetylcholinesterase-derived peptides and uses thereof	US (cip of PCT) PCT/IL00/00311 IL 130224 IL 131707	 31/5/00 30/05/99 02/09/1999	Just filed	2356.03 (7811) 2356.02 2356.00 2356.01

Genetically engineered proteins having human cholinesterase activity	US 5,215,909 (cip App) EU 206 200	18/Jun/1986 18/06/1986	Granted 01/Jun/1993 Granted 23/09/1992	0011.05 0011.03
Hybrid transgenic mouse with accelerated onset of Alzheimer type amyloid plaques in brain (with Mayo Clinic)			Pre-filing	2622.00

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Annex "B"

1. Ohno K, Brengman JM, Felice KJ, Cornblath DR, Engel AG. **Congenital end-plate acetylcholinesterase deficiency caused by a nonsense mutation and an A-->G splice-donor-site mutation at position +3 of the collagenlike-tail-subunit gene (COLQ): how does G at position +3 result in aberrant splicing?** Am J Hum Genet 1999 Sep;65(3):635-44

Congenital end-plate acetylcholinesterase (AChE) deficiency (CEAD), the cause of a disabling myasthenic syndrome, arises from defects in the COLQ gene, which encodes the AChE triple-helical collagenlike-tail subunit that anchors catalytic subunits of AChE to the synaptic basal lamina. Here we describe a patient with CEAD with a nonsense mutation (R315X) and a splice-donor-site mutation at position +3 of intron 16 (IVS16+3A-->G) of COLQ. Because both A and G are consensus nucleotides at the +3 position of splice-donor sites, we constructed a minigene that spans exons 15-17 and harbors IVS16+3A-->G for expression in COS cells. We found that the mutation causes skipping of exon 16. The mutant splice-donor site of intron 16 harbors five discordant nucleotides (at -3, -2, +3, +4, and +6) that do not base-pair with U1 small-nuclear RNA (snRNA), the molecule responsible for splice-donor-site recognition. Versions of the minigene harboring, at either +4 or +6, nucleotides complementary to U1 snRNA restore normal splicing. Analysis of 1,801 native splice-donor sites reveals that presence of a G nucleotide at +3 is associated with preferential usage, at positions +4 to +6, of nucleotides concordant to U1 snRNA. Analysis of 11 disease-associated IVS+3A-->G mutations indicates that, on average, two of three nucleotides at positions +4 to +6 fail to base-pair, and that the nucleotide at +4 never base-pairs, with U1 snRNA. We conclude that, with G at +3, normal splicing generally depends on the concordance that residues at +4 to +6 have with U1 snRNA, but other cis-acting elements may also be important in assuring the fidelity of splicing.

2. Wang Z, Marincola FM, Rivoltini L, Parmiani G, Ferrone S. **Selective histocompatibility leukocyte antigen (HLA)-A2 loss caused by aberrant pre-mRNA splicing in 624MEL28 melanoma cells.** J Exp Med 1999 Jul 19;190(2):205-15

Histocompatibility leukocyte antigen (HLA)-A2 is used as a restricting element to present several melanoma-associated antigen (MAA)-derived peptides to cytotoxic T lymphocytes (CTLs). HLA-A2 antigen is selectively lost in primary melanoma lesions and more frequently in metastases. Only scanty information is available about the molecular mechanisms underlying this abnormality, in spite of its potentially negative impact on the clinical course of the disease and on the outcome of T cell-based immunotherapy. Therefore, in this study we have shown that the selective HLA-A2 antigen loss in melanoma cells 624MEL28 is caused by a splicing defect of HLA-A2 pre-mRNA because of a base substitution at the 5' splice donor site of intron 2 of the HLA-A2 gene. As a result, HLA-A2 transcripts are spliced to two aberrant forms, one with exon 2 skipping and the other with intron 2 retention. The latter is not translated because of an early premature stop codon in the retained intron. In contrast, the transcript with exon 2 skipping is translated to a truncated HLA-A2 heavy chain without the alpha(1) domain. Such a polypeptide is synthesized in vitro but is not detectable in cells, probably because of the low steady state level of the corresponding

mRNA and the low translation efficiency. These results indicate that a single mutational event in an HLA class I gene is sufficient for loss of the corresponding allele. This may account, at least in part, for the high frequency of selective HLA class I allele loss in melanoma cells. Our conclusion emphasizes the need to implement active specific immunotherapy with a combination of peptides presented by various HLA class I alleles. This strategy may counteract the ability of melanoma cells with selective HLA class I allele loss to escape from immune recognition.

3. Chuang JL, Cox RP, Chuang DT. **E2 transacylase-deficient (type II) maple syrup urine disease. Aberrant splicing of E2 mRNA caused by internal intronic deletions and association with thiamine-responsive phenotype.** J Clin Invest 1997 Aug 1;100(3):736-44

Maple syrup urine disease (MSUD) or branched-chain alpha-ketoaciduria is an autosomally inherited disorder in the catabolism of branched-chain amino acids leucine, isoleucine, and valine. The disease is characterized by severe ketoacidosis, mental retardation, and neurological impairments. MSUD can be classified into genetic subtypes according to the genes of the branched-chain alpha-ketoacid dehydrogenase (BCKD) complex which are affected in patients. We describe here four intronic deletions and an intronic nucleotide substitution in the E2 transacylase gene of type II MSUD, in which the E2 subunit of the BCKD complex is deficient. These new E2 mutations comprise an internal 3.2-kb deletion in intron 4 (causing a 17-bp insertion in mRNA), an internal 12-bp (ttaccttgttac) deletion in intron 4 (creating a 10-bp insertion), a 10-bp (catttctaG) deletion in intron 10/ exon 11 junction (leading to a 21-bp deletion), a 2-bp deletion in the exon 5/intron 5 junction (ATgt--> A-t) (resulting in the skipping of exon 5), and a G to A transition at nucleotide -7 of intron 9 (causing a 6-bp insertion). These intronic mutations were initially detected by secondary alterations in the mutant E2 mRNA, as a result of aberrant splicing. The 3.2-kb deletion in intron 4 was determined by the amplification of the entire intron from both a normal subject (11.2 kb) and a homozygous patient (8 kb) by long PCR, followed by subcloning and sequencing of regions flanking the deletion. Similar methods were used to identify and characterize the other intronic alterations. Our results depict heretofore undescribed splicing errors caused by the deletion of internal intronic segments, and provide an approach for detecting this class of novel and rare [illegible] of the type II MSUD patients studied is also discussed.

4. Vervoort R, Buist NR, Kleijer WJ, Wevers R, Fryns JP, Liebaers I, Lissens W. **Molecular analysis of the beta-glucuronidase gene: novel mutations in mucopolysaccharidosis type VII and heterogeneity of the polyadenylation region.** Hum Genet 1997 Apr;99(4):462-8

We used polymerase chain reaction (PCR)/single-strand conformation polymorphism analysis and direct sequencing of the coding region of the beta-glucuronidase cDNA and gene to detect mutations causing beta-glucuronidase enzyme deficiency in five MPS VII patients. Four patients presented with hydrops fetalis, one with an early infantile form of the disease. Genetic heterogeneity of MPS VII alleles was further confirmed in this study. Recurrent mutations were observed in patients of related origin. Previously unknown alleles detected were RII0X, F361delta9, 1270 + 1G-->A, S52F and 1480delta4. Reverse transcription/PCR

analysis of the 1270 + 1G-->A messenger showed aberrant splicing: inclusion of intron 7 or skipping of exons 6-7 and 9. Messenger RNA transcribed from the R110X and 1480delta4 alleles was unstable. We detected a 2154A/G change in the 3' non-coding region of the gene, in the neighbourhood of the two consensus polyadenylation sites. 3'-Rapid amplification of cDNA ends/PCR of fibroblast cDNA revealed equal usage of two alternative polyadenylation sites. The 2154A/G substitution did not influence adenylation-site choice, nor the amount of stable messenger produced. The finding that 2 out of 30 normal controls carried the 2154G allele indicated that the 2154A/G substitution is a harmless polymorphism. The S52F and F361delta9 cDNAs were constructed in vitro and used to transfect COS cells transiently. Both mutations completely abolished enzyme activity.

5. Okubo M, Aoyama Y, Murase T. **A novel donor splice site mutation in the glycogen debranching enzyme gene is associated with glycogen storage disease type III.** Biochem Biophys Res Commun 1996 Jul 16;224(2):493-9.

Analysis of glycogen debranching enzyme (debrancher) cDNA from a patient with glycogen storage disease type III revealed a deletion of 124 base pairs. A donor splice site mutation (IVS G+1 to T) was identified in the patient's debrancher gene, which caused exon skipping of the upstream exon and resulted in a truncated enzyme due to premature termination. Mutational analysis of the patient's family showed that this point mutation was inherited from the father. Southern blot analysis of the patient's genomic DNA showed an additional, unique EcoRI fragment of 5.8 kb, which was inherited from the mother. These results suggested that the patient was a compound heterozygote for the donor splice site mutation, which is the first identified in the debrancher gene, and had a genetic defect relating to an aberrant 5.8-kb EcoRI fragment.

6. Matsuura T, Hoshide R, Komaki S, Kiwaki K, Endo F, Nakamura S, Jitosho T, Matsuda I. **Identification of two new aberrant splicings in the ornithine carbamoyltransferase (OCT) gene in two patients with early and late onset OCT deficiency.** J Inherit Metab Dis 1995;18(3):273-82Related Articles,

Ornithine carbamoyltransferase (OCT) is a liver-specific enzyme located in the mitochondrial matrix. OCT deficiency is an X-linked disease with a heterogeneous phenotype, even in affected males. We studied two male patients (K.M., K.G.) with early and late onset, respectively. OCT activity was zero in the autopsied liver of patient K.M. and was 6% of control in the biopsied liver of K.G. Sequencing of OCT cDNAs revealed exon 5 skipping in K.M., resulting from a T-to-C transition of the initial dinucleotide of the 5' splicing donor site of intron 5, and a G-to-T transversion at position +45 in exon 9 (L304F) in K.G., providing three OCT mRNAs of different lengths: a normally spliced transcript, 23 bp insertion of intron 8 and the first 50bp missing within exon 9. Exon 5 skipping and two other aberrant splicings produced stop codons early downstream in mature OCT mRNAs. Expression study of a missense allele, L304F, transfected to cultured Cos 1 cells revealed a 34.4% value of the control. The difference of OCT activities between the patient liver and transfected cells (6% vs. 34%) can be explained by this splicing abnormality.

7. Nakai K, Sakamoto H. **Construction of a novel database containing aberrant splicing mutations of mammalian genes.** Gene 1994 Apr 20;141(2):171-7

To explore the rules for mammalian splice-site selection using a statistical approach, we constructed an aberrant splicing database containing an extensive collection of mammalian genetic disease mutations (90 genes, 209 mutations). From this database, we confirmed that: (1) more than 90% of mutations either destroy or create the splice-site consensus sequences; (2) the number of mutations mapped at individual residues in the splice-site regions roughly correlates to their conservation degrees in the consensus sequences; (3) about half of the observed aberrant splicing is exon skipping, while intron retention is rarely observed; (4) almost all of the major cryptic sites, activated by mutations, are mapped within an about 100-nt region from the authentic splice sites. Furthermore, we found that: (5) mutations are observed more frequently in the 5' splice-site region than in the 3' splice site region; (6) splice sites that are newly created by mutations are located upstream from the authentic splice sites. Hopefully, these observations will be used as rules for constructing a more effective prediction system of exon sequences.

8. Lemmink HH, Kluijtmans LA, Brunner HG, Schroder CH, Knebelmann B, Jelinkova E, van Oost BA, Monnens LA, Smeets HJ. **Aberrant splicing of the COL4A5 gene in patients with Alport syndrome.** Hum Mol Genet 1994 Feb;3(2):317-22

A variety of mutations have been identified in the X-linked type IV collagen alpha 5 chain (COL4A5) gene in patients with Alport syndrome. A substantial number of these mutations were predicted to have an effect on RNA splicing. For 4 such mutations in our group of patients the effect of the DNA mutation on the COL4A5 mRNA structure and stability was analysed. An alteration of the invariant splice acceptor site of intron 41 resulted in a shift of the actual splicing to either a cryptic splice site within exon 42 or the normal splice site in the next intron. A single base substitution of the final nucleotide of exon 48 resulted in the removal of the entire exon. Two frameshift mutations, a 10 basepair duplication in exon 49 and a single base deletion in exon 49, were [illegible] RNA as such and resulted in a stretch of missense codons terminated by a premature stop codon. Exon skipping was occasionally observed in these samples, but not reproducibly in every experiment. In healthy controls exon skipping was never detected. Analysis of female carriers revealed that in only one case was the stability of the mutated mRNA reduced in comparison with the normal transcript. The extent to which the non-collagenous domain was predicted to be deleted correlated with the severeness of the disease.

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REVIEW

Pre-mRNA splicing modulations in senescence

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Summary

Aging and associated diseases involve multilevel changes in the complex phenomenon of alternative splicing. Here, we review the potential genomic and environmental origins of such changes and discuss the research implications of these findings.

Key words: aging; alternative splicing; pre-mRNA; SR proteins; hnRNPs.

Introduction

From the viewpoint of a molecular biologist, aging reflects gradual deterioration of the molecular components, checkpoints and/or events, the concerted functioning of which are vital for cell viability and proliferation. The complexity of alternative splicing of pre-mRNA makes this process particularly vulnerable to senescence, leading to both transient changes and chronic aging-related diseases.

With the emergence of microarray technologies, changes in gene expression profiles during aging have recently become the focus of extensive research. However, relatively little is yet known about changes in alternative splicing, a key pre-mRNA processing mechanism during cellular and organismal senescence.

The progressive nature of the aging process is often ascribed to passive accumulation of late-onset mutations due to lack of natural selection beyond the age of reproduction (Kirkwood, 2002). Alternatively, aging is viewed as the outcome of active selection of traits that are beneficial at an early stage of life but have deleterious effects later on (reviewed in Partridge & Gems, 2002). The idea that aging is a genetically programmed mechanism has also been subject to some debate. Planned or sporadic, passive or active, aging also involves changes in the pathways of gene expression that are not necessarily associated with mutation. This is exemplified in most aging-related diseases, such as Alzheimer's and Parkinson's diseases and tauopathies, which represent the best studied cases of aging, involving aberrations in the alternative splicing of pre-mRNA. Here, we describe this emerging concept and discuss its research implications. We present examples of alternative splicing events during aging, and propose that these examples reflect a generally modified state of the pre-mRNA processing machinery in senescent cells, leading to altered expression levels of pre-mRNA processing factors and their downstream target mRNAs and corresponding proteins during aging.

Aging-related modifications in the alternative splicing of specific gene products were sought, primarily as a means to search for the molecular origin(s) of aging-related deterioration, for example, neural adhesion processes, dopaminergic neurotransmission, insulin responses and gastric cytoprotection (Table 1).

Together, the picture that emerges from these studies is of a general control switch that causes all of these aging-induced impairments in alternative splicing. For example, these may all reflect one or a few changes in regulatory factor(s), such as splicing-related proteins, which modify the delicate regulation of the splicing process. Support for this concept can be found in recent microarray screens for changes in gene expression during aging (Lee *et al.*, 1999; Shelton *et al.*, 1999; Cao *et al.*, 2001; Tollet-Egnell *et al.*, 2001). Significant age-related tissue-specific changes were found in the expression levels of several RNA processing genes in aged, compared to young, gastrocnemius muscle, neocortex and cerebellum of C57BL/6 mice (Lee *et al.*, 1999). Changes spanned serine, arginine-rich (SR) proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs), as well as 3′-end processing factors (Table 2). We present a scheme of the key processes in the pathway of pre-mRNA splicing (Fig. 1), highlighting those elements in which age-related changes have been reported.

Pre-mRNA processing during aging

SR proteins contain at least one RNA recognition motif (RRM) and an arginine, serine-rich (RS) domain, allowing them to bind both the pre-mRNA and additional proteins. They bind the nascent pre-mRNA at specific sites, which can act as both splicing enhancers and splicing suppressors. These *cis*-acting motifs are usually degenerate and can be found within both exons and introns, exonic splicing enhancers (ESEs) being the more prevalent. Through such interactions with the pre-mRNA, SR proteins can regulate splicing and alternative splicing in a concentration-dependent manner (Manley & Tacke, 1996).

hnRNPs serve as a crucial checkpoint in pre-mRNA processing. They include a diverse group of proteins containing RNA binding motifs as well as several auxiliary domains. This allows them to simultaneously bind pre-mRNA and other proteins (Krecic & Swanson, 1999). Misdirected regulation of the expression levels

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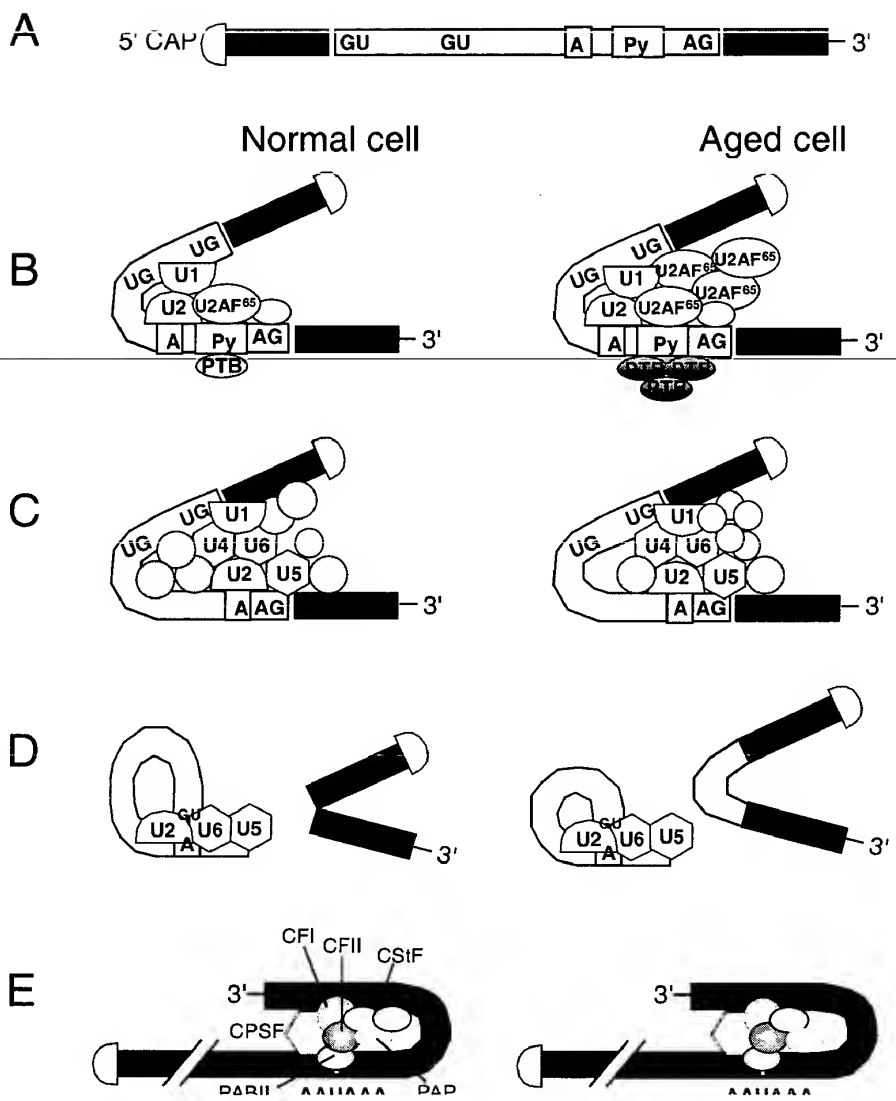

Fig. 1 Misdirected regulation of pre-mRNA processing during aging. Factors reported to change in level during aging are depicted, along with the different steps of pre-mRNA processing in which they are involved in normal and aged cells. The representations are not to scale; only one intron is shown, and in E, the spliced exons are extended to allow representation of the cleavage and polyadenylation factors. A. The 'naked' pre-mRNA transcript. Exons are marked red and introns white. Shown are two alternative 5′ (GU) splice sites and a single 3′ (AG) splice site, the branch point (A) and the polypyrimidine tract (Py). The 5′ end of the pre-mRNA is capped shortly after initiation of transcription. (The 3′ end, A–D, does not represent the physical end of the pre-mRNA). B. Initial snRNP complex formation. The U1 small nuclear RNA and associated proteins comprise the U1 snRNP, which binds the 5′ splice site. Likewise, the U2 snRNA forms snRNP complexes, which are recruited to the branch point by U2AF. U2AF binds the Py tract through its heavy ($U2AF^{65}$) subunit. PTB also binds the Py tract and regulates splicing. $U2AF^{65}$ and PTB were shown to be significantly over-produced in the gastrocnemius muscle and cerebellum, respectively, of aged CS7BL/6 mice, thus potentially affecting splicing (the stoichiometry presented is speculative). C. Spliceosome assembly and attraction of SR proteins. The remaining spliceosomal components (U4, US, U6) assemble together with additional SR proteins, shaded grey. These splicing factors bind simultaneously exonic or intronic splicing enhancer sequences on the pre-mRNA through their RRM and additional proteins through their R5 domain. Several of these components were shown to be modified in aged tissues. The splicing-related factor/RNA helicase PRP16, for example, was ca. two-fold decreased in the cerebellum. D. Intron release and exon joining. Splicing involves two sequential transesterification reactions, which result in the release of the intron in the shape of an RNA 'lariat', and ligation of the flanking exons to form the mRNA. Altered expression levels of the participating factors may lead to the alternatively spliced transcript shown. E. 3′ end processing. The cleavage and polyadenylation complex is shown. This complex consists of CPSF, which recognizes and binds the poly(A) site (AAUAAA), together with CStF, CFI, CFII and PAP, the combined complex of which catalyses the first, slow, polyadenylation step. PABII then binds the short, newly formed, poly(A) tail and rapidly adds additional 200–250 adenines. CStF and PAP were both ca. 1.5-fold decreased in the neocortex of CS7BL/6 aged mice, potentially harming the 3′-end processing pathway. Abbreviations: Py, polypyrimidine tract; snRNP, small nuclear ribonucleoprotein particle; U2AF, U2 auxiliary factor; PTB, polypyrimidine tract binding protein; SR-proteins, serine, arginine-rich proteins; CPSF, cleavage and polyadenylation specificity factor; CStF, cleavage stimulation factor; CF, cleavage factor; PAP, poly(A) polymerase; PAB, poly(A)-binding protein.

Table 1 Aging-associated changes in alternative splicing

Gene	Organism	Tissue	Affected process	Reference
APP, APLPs	rat	brain	amyloid plaque formation	(Sandbrink *et al.*, 1997)
Dopamine D2 receptor	rat	neostriatal subregions	dopaminergic neurotransmission	(Merchant *et al.*, 1993)
N-CAM	rat	heart	neural adhesion	(Andersson *et al.*, 1993)
Insulin receptor	rat	liver, muscle, heart	insulin responses	(Vidal *et al.*, 1995)
MGF	rat	skeletal muscle	insulin responses	(Owino *et al.*, 2001)
COX-1	rat	stomach	gastric cytoprotection	(Vogiagis *et al.*, 2000)
NF1	human	blood	rRas signalling, neural architecture	(Wimmer *et al.*, 2000)

Table 2 Aging-associated alterations in the expression of murine pre-mRNA processing genes

Gene	Fold change*	Tissue	Affected pre-mRNA processing step	Reference to reported function
SF3A2 (SAP62, PRP11)	3.4	cerebellum	U2 snRNP binding	(Ruby *et al.*, 1993)
$U2AF^{65}$	3.2	gastrocnemius muscle	Splice site recognition, splicing	(Wang *et al.*, 1995)
Sox17	2.4	gastrocnemius muscle	Transcription, splicing	(Ohe *et al.*, 2002)
hPRP22 (HRH1)	2.5	neocortex	Spliceosome disassembly, 2nd catalytic step, mRNA release	(Company *et al.*, 1991); (Schwer & Gross, 1998)
PTB	2.3	cerebellum	Alternative splicing	(Wagner & Garcia-Blanco, 2001)
PolyA + RNA export protein	2.1	gastrocnemius muscle	3′ end processing, mRNA export	
hnRNP H3 (hnRNP 2H9)	2.0	cerebellum	Splicing, heat-shock splicing arrest	(Mahe *et al.*, 1997)
CStF	0.7	neocortex	Transcript cleavage	
DDX18	0.7	neocortex	DEAD-box protein, specific function unknown	
hPRP16	0.6	cerebellum	Aberrant lariat discard	(Burgess & Guthrie, 1993)
PABI	0.4	cerebellum	mRNA stability	
hPRP22	0.4	cerebellum	Spliceosome disassembly, 2nd catalytic step, mRNA release	(Company *et al.*, 1991); (Schwer & Gross, 1998)

*From Lee *et al.* (1999).

of SR-proteins and hnRNPs can influence splice site selection or lead to alternative or aberrant splicing of many downstream target sequences. hnRNP A1, for example, was shown to suppress splicing of exon 3 of the HIV-1 tat gene through high-affinity binding to an exonic splicing silencer (ESS) and recruitment of additional hnRNP A1 molecules, while the SR protein ASF/SF2 prevents this hnRNP A1 accumulation at the ESS and antagonizes the splicing arrest (Zhu *et al.*, 2001).

One of the first essential splicing factors to be identified was the 65-kDa subunit of the U2 auxiliary factor ($U2AF^{65}$). $U2AF^{65}$ binding to the conserved poly pyrimidine (Py) tract at the 3′ end of introns is required for the subsequent binding of U2 snRNA to the 5′ splice site (usually a GU dinucleotide). The other subunit, $U2AF^{35}$, binds the 3′ splice site (usually an AG). $U2AF^{65}$ mRNA levels were found to increase by over three-fold in the gastrocnemius muscle of aged mice (see References cited in Table 2). As $U2AF^{65}$ contains both the RNA recognition motif and the RS domain of SR-proteins, it can regulate splicing in a concentration-dependent manner, as do the SR proteins (Manley & Tacke, 1996). Uncontrolled regulation of the expression level of $U2AF^{65}$ may therefore lead to aberrant splicing activity and alternative splicing of numerous target genes (Wang *et al.*, 1995).

The Py tract-binding protein (PTB, hnRNP I) also binds intronic Py tracts. PTB was shown to play a direct role in pre-mRNA alternative splicing through antagonistic effects on exon definition, repressing, for example, the inclusion of exon 7 in the β-tropomyosin gene (Wagner & Garcia-Blanco, 2001). Its expression levels were reported to increase over two-fold in the cerebellum of aged mice, which can potentially harm finely regulated alternative splicing of many pre-mRNAs by repressing exon inclusion and promoting aberrant splicing.

Other factors that may have a direct or indirect role in influencing splicing during aging are hPRP22, hPRP16, SF3A2, DDX18 and hnRNP H3 (Table 2). The expression level of the splicing factor PRP22, for example, was 2.5-fold increased in the neocortex of aged C57BL/6 mice, while the same factor displayed 2.7-fold decrease in the cerebellum of these mice (Lee *et al.*, 1999). Although the function of several of these factors is largely obscure, this expanded list points to a central involvement of splicing changes in the aging process.

Initiation and efficacy of transcription

Another checkpoint, which may affect aging-related changes in pre-mRNA splicing, occurs prior to the splicing process, at the earlier phases controlling the initiation and efficacy of transcription. This involves the expression levels of a large number of transcription factors, also shown to be modified in aged tissues (Lee *et al.*, 1999; Cao *et al.*, 2001; Tollet-Egnell *et al.*, 2001). Since transcription and splicing are tightly coupled

processes, such changes are likely to affect the splicing machinery as well. The transcription factor SOX17 (D49473), for example, with a direct role in pre-mRNA splicing (Ohe *et al.*, 2002), exhibited 2.4-fold over-expression in the aged gastrocnemius muscle. While changes in expression levels may not correlate directly with changes in activity, altered expression of transcription-associated genes during aging adds further support to the concept of pre-mRNA splicing as an age-sensitive process. Indeed, aging-related diseases often reflect abnormal upstream factors, including impaired regulation or aberrant fine-tuning of mRNA processing in general and pre-mRNA splicing in particular.

The 3′-end processing complex of pre-mRNA is also subject to aging-related changes. This complex consists of cleavage factors 1 and 2 (CF1, 2), cleavage stimulation factor (CStF), cleavage and polyadenylation specificity factor (CPSF), poly(A)-polymerase (PAP), and poly(A)-binding protein II, which acts after the initial, slow polyadenylation phase is complete (Minvielle-Sebastia & Keller, 1999). CStF was decreased in the neocortex of aged mice (Lee *et al.*, 1999), perhaps indicating a depression of the 3′-terminal processing of nascent mRNAs in conjunction with the aberrant splicing that accompanies aging. The cytoplasmic poly(A)-binding protein (PABI) was decreased to less than half its normal level in the cerebellum of aged mice, while the poly(A)+export protein was similarly increased in the gastrocnemius muscle of these mice. This points to the tissue specificity of age-dependent changes in 3′-end processing and mRNA export, again, keeping in mind that changes in expression levels may not necessarily directly correlate with changes in activity.

Age-related diseases

Age-related, late-onset diseases are especially vulnerable to splicing variations of different origins. These include mutations affecting the correct splicing of a particular gene. Age-related macular degeneration (AMD) is an example of an aging-related disease that is associated with a late-onset splicing mutation (Allikmets *et al.*, 1997). AMD is the most common cause of acquired visual impairments in the elderly (Stone *et al.*, 2001). The occurrence of AMD is often associated with mutations within the Stargardt disease gene (STGD1 or ABCR), coding for a photoreceptor-specific member of the ATP-binding cassette (ABC) superfamily of transporter proteins. One of these mutations, a G for A substitution at position 5196, was found to be a donor splice site mutation (Allikmets *et al.*, 1997). The late onset of the disease phenotype, in this case, may be explained by age-related modifications in the efficacy and/or composition of pre-mRNA processing factors, since the disease is associated with the consequences of a genomic splice-site mutation that for unknown reasons are apparent only at a later stage in life.

Aging-related impairments in pre-mRNA processing need not necessarily involve a protein-modifying mutation as they may be caused by an altered upstream splicing regulator. Amyotrophic lateral sclerosis (ALS) serves as an example.

ALS is a late-onset neurodegenerative disease that is characterized by selective degeneration of spinal cord motor neurones. Most ALS patients suffer from a significant loss of the astroglial excitatory amino acid transporter 2 (EAAT2, previously known as GLT-1) in the motor cortex and spinal cord (Lin *et al.*, 1998). Affected tissues are highly populated by abnormal EAAT2 mRNA species resulting from aberrant alternative splicing. Since the EAAT2 gene is not mutated in these patients, it was suggested that a protein involved in pre-mRNA processing is the culprit (Bai & Lipton, 1998). Furthermore, alternative splicing of neuronal nitric oxide synthase (nNOS) was demonstrated in the spinal cord of ALS patients: nNOSβ and nNOSγ, but not nNOSα, were up-regulated (Catania *et al.*, 2001). This demonstrates modified alternative splicing of nNOS, in addition to EAAT2, and strengthens the hypothesis that an as yet unknown aberrant upstream splicing regulator is involved in ALS pathology.

Alzheimer's disease (AD) is the most common neurodegenerative disorder of aging, characterized by progressive memory loss and cognitive deterioration. AD involves premature death of selected cholinergic neurones, associated with the formation of amyloid plaques, the appearance of which in the brain of patients is facilitated by mutations in several different genes (e.g. APP, PS1, PS2). Misdirected splicing regulation of relevant gene products either due to specific mutations or to aberrant processing of such products with no known mutation were both demonstrated in AD. Presenilins provide examples of both types of alteration: mutations within the fourth intron of presenilin 1 (PS1) were shown to impair PS1 splicing and cause early onset AD, while an exon 5-deficient splice variant of apparently normal PS2, which accumulates following hypoxia in cultured neuroblastoma cells, was found to be prevalent in the brains of AD patients, indicating that this unique splice variant is inducible, rather than being produced at a constant level due to mutation in the corresponding gene (Sato *et al.*, 1999).

Tauopathies are a family of late-onset neurodegenerative diseases associated with mutations within the microtubule-associated protein (MAP) tau gene (Lee *et al.*, 2001). Progressive accumulation of filamentous tau inclusions causes neural degeneration in specific brain regions of patients with tauopathies. The late onset of this phenotype suggests that an age-related molecular change is responsible. Tau is alternatively spliced in the adult human brain (Lee *et al.*, 2001) and transgenic mice over-expressing the shortest human tau variant display age-dependent CNS deterioration reminiscent of human tauopathies (Ishihara *et al.*, 1999). Thus, as yet unspecified impairments in pre-mRNA processing and/or splicing may contribute to these neurodegenerative conditions.

Frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) is an example of a tauopathy associated with changes in alternative splicing. Several mutations within the tau gene were demonstrated for FTDP-17, a large proportion of which affect the splicing pattern of tau exon 10 by influencing exonic/intronic splicing enhancers/suppressors (D'Souza *et al.*, 1999). The aberrant splicing of tau exon 10 alters the ratio of the tau isoforms incorporated into the neuronal tangles that are the neuropathological hallmark of the demented brain. Excessive tau accumulation results in filamentous inclusions

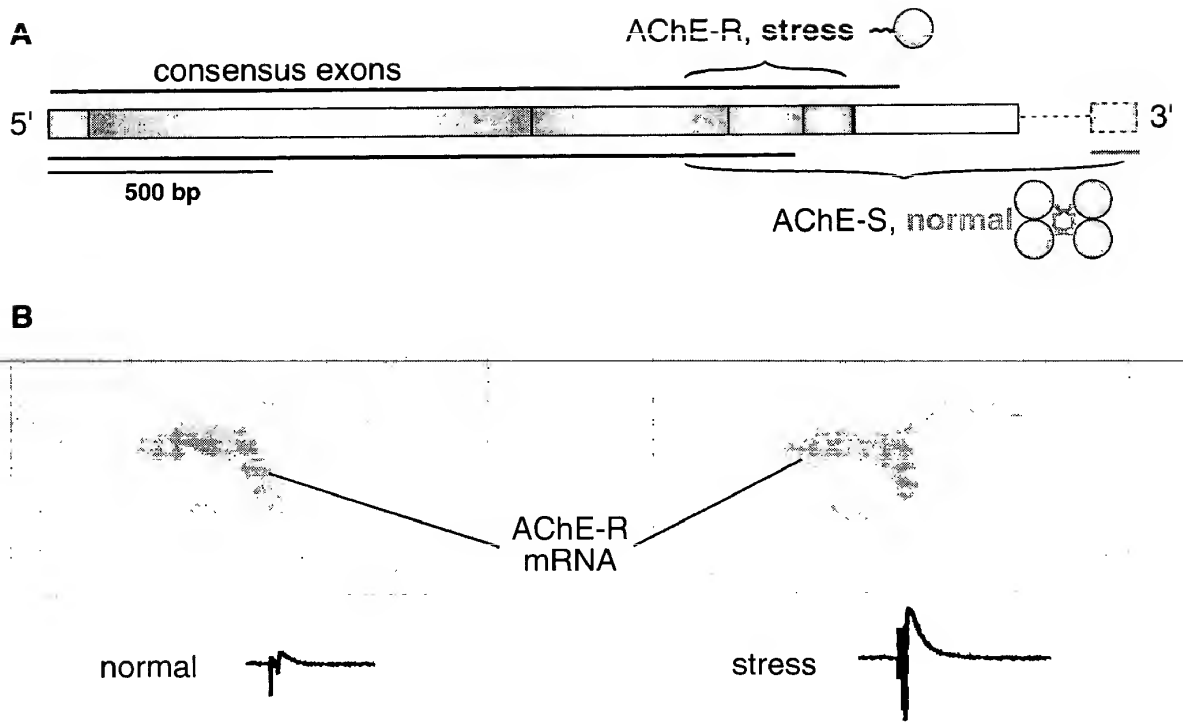

Fig. 2 Stress-induced alternative splicing in neuronal acetylcholinesterase mRNA. A. The AChE mRNA transcript includes 5′-consensus exons as well as variant-3′ exons, which are subject to alternative splicing under the influence of various psychological, physical and chemical stressors (Soreq & Seidman, 2001; Meshorer *et al.*, 2002). The primary transcript (normal) encodes a protein with a C-terminal sequence, which enables multimerization and binding to a non-catalytic structural subunit, allowing attachment to the synaptic membrane. The stress-induced alternative transcript encodes a protein with a distinct C-terminal sequence, which does not allow multimerization, leading to the soluble, secretory isoform. B. Under normal conditions, AChE-R mRNA resides in the perinuclear area, but stress induces its dendritic translocation. The AChE-R protein product fails to adhere to the synaptic membrane, resulting in exaggerated field potentials under stress, measured as extracellular recordings on the CA1 areas of hippocampus slices in response to stimulation of stratum oriens, shown here as representative voltage traces (Meshorer *et al.*, 2002).

within these tangles. In this case as well, the mutated genotype leads to an abnormal phenotype with a delayed onset, hinting at a change in an unidentified age-related factor involved in pre-mRNA processing.

Stress and splicing

Another consideration with regard to late-onset phenotypes involves the interrelationships between individual genotypes and the environment. Stress-induced changes in the pathway to gene expression, for example, may change the age of onset of an aberrant pattern of pre-mRNA splicing, initiating a cascade of events that expedite the onset of an aging-related disease. Such conditions are evident primarily in the central nervous system because of its role in the initiation of stress responses and because of the range of cell types and complex gene expression profiles in the mammalian brain. Examples include the aberrant splicing of K^+ channels (Xie & McCobb, 1998), of the Tra1 splicing regulator (Daoud *et al.*, 1999) and of acetylcholinesterase (AChE) mRNA (Meshorer *et al.*, 2002). The latter example is particularly relevant to the issue at hand, as it directly relates to the behavioural changes and the cognitive impairments which are characteristic of old age and which are implicated in age-related neurodegenerative diseases.

The *ACHE* gene gives rise to several mature transcripts. Of these, the primary 'synaptic' AChE-S mRNA constitutes the preponderant AChE mRNA under normal conditions and is translocated into neuronal processes. Under the influence of various stressors, however, a splicing shift occurs which leads to overproduction and neurite translocation of the normally rare 'readthrough' AChE-R mRNA transcript (Fig. 2). It is not yet clear why AChE-R mRNA stays in the cell body under normal conditions but travels to neuronal processes under conditions of stress. However, this change alters both the nature of the AChE protein product and its subcellular location (Fig. 2). Thus, neuritic AChE-S forms synaptic membrane-associated tetramers; in contrast, AChE-R forms soluble, secretory monomers that cannot adhere to the membrane and hence fail to address incoming cholinergic stimuli, leading to prolonged neuronal hypersensitivity (Meshorer *et al.*, 2002). Large excesses of AChE-R, such as those that accumulate in the injured brain, are detrimental: massive overproduction of this variant in the brain of head-injured transgenic mice over-expressing human AChE reduces survival and slows the recovery, whereas antisense intervention with AChE-R

production improves survival and recovery of both transgenic mice and their parent strain (reviewed in Soreq & Seidman, 2001). As head injury is the largest risk factor known for non-familial AD, the splice shift leading to AChE-R overproduction seems to be causally involved in an increased risk for neurodegeneration. That the AD brain displays increases in AChE-R monomers (Darreh-Shori *et al.*, in press) as opposed to decreases in AChE-S tetramers supports this notion.

Cell proliferation

One final key process, which is now known to be affected by aging and which depends on the regulation of pre-mRNA processing, is cell proliferation. Pre-mRNA processing events, which are subject to aging dependent impairment, participate in control of cell proliferation (Verdi *et al.*, 1999), and aging has long been known to be associated with aberrant cell proliferation (Cameron, 1972; Xiao *et al.*, 2001). Therefore, although neuronal stem cells maintain into old age some capacity to proliferate (van Praag *et al.*, 2002), the efficacy of cell proliferation is likely affected by damaged splicing, with consequences for many physiological functions. The proliferation of neuronal stem cells in the murine hippocampus, for example, emerges as an essential prerequisite for the preservation of newly acquired trace memories (Shors *et al.*, 2001). This physiological process is impaired even in non-demented, normal older individuals (West & Covell, 2001). Our current considerations therefore imply that the memory impairments of old age may reflect detrimental changes in pre-mRNA processing and consequent impairment in stem cell proliferation.

Most of the examples presented above refer to post-mitotic tissues such as brain and muscle, in which the relevant cells are terminally differentiated. In view of this line of speculation, it would be most interesting to explore the changes during aging that take place in the pre-mRNA processing machinery of proliferating cells of these organs, particularly of the neuronal stem cells.

Concluding remarks

Because microarray screening for age-related changes will identify genes and regulatory factors only if there happens to be a substantial change in their expression, advances by this approach alone may be limited. Based on the evidence we have presented, advances in the molecular biology of aging may well come about by screening for alternative splicing options of key genes that encode factors that contribute to pre-mRNA processing.

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